Altered visual processing in a rodent model of Attention Deficit Hyperactivity Disorder

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Abstract

A central component of Attention Deficit Hyperactivity Disorder (ADHD) is increased distractibility, which is linked to the superior colliculus (SC) in a range of species, including humans. Furthermore, there is now mounting evidence of altered collicular functioning in ADHD and it is proposed that a hyper-responsive SC could mediate the main symptoms of ADHD, including distractibility. In the present study we have provided a systematic characterisation of the SC in the most commonly used and well-validated animal model of ADHD, the spontaneously hypertensive rat (SHR). We examined collicular-dependent orienting behaviour, local field potential (LFP) and multiunit responses to visual stimuli in the anaesthetised rat and morphological measures in the SHR in comparison to the Wistar Kyoto (WKY) and Wistar (WIS). We found that the SHR remain responsive to a repeated visual stimulus for more presentations than control strains and have longer response duration. In addition, LFP and multiunit activity within the visually responsive superficial layers of the SC showed the SHR to have a hyper-responsive SC relative to control strains, which could not be explained by altered functioning of the retinocollicular pathway. Finally, examination of collicular volume, neuron and glia densities and glia:neuron ratio revealed that the SHR had a reduced ratio relative to the WKY which could explain the increased responsiveness. In conclusion, this study demonstrates strain-specific changes in the functioning and structure of the SC in the SHR, providing convergent evidence that the SC might be dysfunctional in ADHD.

Keywords: Superior colliculus; Spontaneously Hypertensive Rat; Distractibility; Orienting

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is the most common neurodevelopmental disorder, affecting 8–12% of children (Biederman and Faraone, 2005), with symptoms often persisting into adulthood (Spencer et al., 2002). It is characterised by difficulty with attention, impulsivity and hyperactivity. A central component of ADHD is an increase in distractibility (Douglas, 1983, Thorley, 1984), which has long been considered one of the most common symptoms of ADHD (Barkley and Ullman, 1975) and features in the inattentive and combined presentations of ADHD under DSM-5 (APA, 2013).

Behavioural evidence suggests that distractibility is intimately linked with the superior colliculus (SC), a subcortical structure that is highly conserved across species (Ingle, 1973). The SC is involved in detecting and responding to novel, unexpected and salient stimuli across a range of modalities (Dean et al., 1989). In particular, it is responsible for orienting head and eye movements (Grantyn et al., 2004) and covert attention towards such stimuli (Rizzolatti et al., 1987). Work in a range of species has shown that collicular lesions cause a decrease in distractibility (Sprague and Meikle, 1965, Goodale et al., 1978, Milner et al., 1978) whilst removal of prefrontal cortex inhibitory control of the colliculus leads to an increase in distractibility in humans (Gaymard et al., 2003). This suggests that the SC remains important in the neural basis of distractibility in humans.

Although many theories have been proposed about the underlying neural basis of ADHD, it is still poorly understood (Biederman, 2005). Theories include frontal cortex deficits (Barkley et al., 1992) and/or alterations in monoamine transmission, particularly dopaminergic function (Wender, 1973). However, several lines of evidence support a role for the SC in ADHD. Firstly, people with ADHD have difficulty inhibiting saccades (Klein et al., 2003, O'Driscoll et al., 2005) and shifts in covert attention (Swanson et al., 1991), consistent with

collicular dysfunction (Ignashchenkova et al., 2004, Katyal et al., 2010, Robinson and Bucci, 2014). Secondly, collicular dysfunction has been reported in rodent models of ADHD. For example, in the spontaneously hypertensive rat (SHR), the most commonly used rodent model of ADHD, altered height dependency of air righting reflexes has been found (Dommett and Rostron, 2011) which is linked to collicular dysfunction (Pellis et al., 1989, Pellis et al., 1991, Yan et al., 2010). More recently, orienting behaviour to a repeated visual stimulus has been shown to be increased in the SHR (Robinson and Bucci, 2014). In addition, in the New Zealand Genetically Hypertensive (GH) rat, a proposed, but as yet not widely validated model of ADHD, increased responsiveness to whole field light flashes has been found in the superficial layers of the colliculus (Clements et al., 2014). Thirdly, amphetamine which is used to treat ADHD, decreases the responsiveness of cells in the superficial layers of the colliculus to visual stimuli in healthy rats (Gowan et al., 2008) and the New Zealand GH rat (Clements et al., 2014). It also reduces distractibility in healthy rats (Agmo et al., 1997) and humans both with (Brown and Cooke, 1994, Spencer et al., 2001) and without ADHD (Halliday et al., 1990). Finally, the colliculus is known to modulate ascending dopaminergic systems (Dommett et al., 2005) via a direct connection from the colliculus to midbrain dopaminergic neurons (Coizet et al., 2003, Comoli et al., 2003) and, therefore, alterations in collicular functioning could cause the dopaminergic abnormalities seen in ADHD (Solanto, 2002, Viggiano et al., 2003a, Viggiano et al., 2003b, Sagvolden et al., 2005).

In light of the mounting evidence supporting a role for the SC in ADHD, we conducted a detailed characterisation of the SC, focusing on the visually-responsive superficial layers, in the SHR model of ADHD. Despite previous studies suggestive of a collicular abnormality in ADHD, no study to date has utilised evidence from behavioural, physiological and morphological techniques within a validated animal model. Specifically, we hypothesized that the SHR would show increased responsiveness to visual stimuli both at a behavioural

level on an orienting task and at neuronal level in the colliculus. Furthermore, we hypothesized that there would be changes in the underlying morphology (collicular volume, cell densities and neuron-glia ratio) of the colliculus.

2. Methods and materials

2.1 Animals

All experiments were conducted with the authority of the appropriate UK Home Office Licenses and adhered to guidelines set out in the Animals [Scientific Procedures] Act (1986), EU Directive 2010/63/EU, and the "Guide for the care and use of Laboratory Animals" (NIH publication, 8th ed, The National Academies Press, Washington, 2011). Adult male rats (Harlan Laboratories Ltd, Bicester, UK) aged 15-20 weeks at the start of testing were housed within the Biomedical Resource Unit (BRU) at the Open University. All rats were housed in groups of three (of the same strain) within scantainers held at a constant temperature of 21-23 °C. The holding room was on a 12:12hr reverse light/dark cycle with lights off at 8am. Rats were given one week to habituate to the BRU prior to use in any experimental procedures. All procedures were carried out in the dark phase and therefore at the time when rats are most active. Food and water were available ad libitum throughout. The importance of an appropriate control strain for the SHR is widely recognised (Sagvolden et al., 2009), and as such, we selected both the Wistar Kyoto (WKY), the normotensive control commonly used but also shown to have some abnormal behaviours in itself (Drolet et al., 2002, van den Bergh et al., 2006), and the Wistar (WIS) as an outbred albino control strain. The sample sizes and weight in grams at start of data collection for the different experimental procedure for the three strains is shown in Table 1.

Table 1 The division of animals across the different experimental procedures giving their weight in grams (mean ± SEM) and sample size. Note that animals used for behavioural testing were also used for electrophysiology experiments, but the animals used for the morphological measures were solely used for this purpose. The total number of animals used was therefore 115.

Despite the animals of each strain being the same age at the start of the data collection phases for each of the experimental procedures, there were significant differences in weight between strains for the behavioural ($F(2)=8.39$; $p=0.002$) and physiological measures ($F(2)=28.19$; $p=0.0005$). In both cases, post hoc (Tukey HSD) analysis revealed that the only significant differences were between the WIS and the WKY (behaviour $p=0.002$; physiological $p=0.0005$) and the WIS and the SHR (behaviour $p=0.026$; physiological $p=0.0005$), with the WIS weighing more than both other strains. There was no significant difference in weight between the strains for the morphological procedures (volume $F(2)=3.14$; $p=0.092$; cell densities and ratios $F(2)=1.33$; $p=0.311$).

2.2 Behavioural testing

Distractibility was measured using an orienting task with a visual stimulus, examining initial responses and subsequent habituation of the response to the visual stimulus (Clements et al., 2014, Robinson and Bucci, 2014). All testing was carried out between the hours of 9am and 5pm in a dimly red-lit room in the presence of white noise and with careful removal of olfactory cues from testing equipment between test sessions to remove any extraneous cues that could affect behaviour. Prior to testing, animals were habituated to the experimenter with daily handling for one week. In addition, they were habituated to the testing space, a circular plastic arena (2.5 m diameter) with a centrally located light (green LED, 20 mcd) sealed within a clear Perspex cylinder, for two days prior to testing. On each habituation day the animal was placed in the arena for 15 minutes with the stimulus light remaining off for the entire period. Testing began on the third day with the animal placed in the arena and the video camera started (Samsung VP-HMX20C). After 5 minutes, the light was remotely

switched on for a period of 5 seconds. This was repeated for a total ten stimulus presentations with an inter stimulus interval of 5 minutes. Order of testing was counterbalanced by strain and the remote control of the paradigm meant that the experimenter was not present in the room during testing and therefore could not influence behaviour.

Offline video analysis was used to determine whether an animal had oriented to the stimulus. An animal was deemed to have oriented if it physically interacted with the stimulus casing, oriented its head towards the stimulus or stared at the stimulus. Once it was determined whether the animal had responded, it was possible to calculate the percentage of animals of each strain that responded for each of the ten consecutive stimulus presentations. The comparison of interest was between strains and therefore a survival analysis was used to assess whether any difference in responsiveness across repeated stimulus presentation was significant. We examined whether there were any strain differences in the median survival time i.e. the number of stimulus presentations before which 50% of those rats initially responding showed habituation. In addition to whether a response occurred, the duration of any response to the stimulus during the 5 seconds in which it was on was measured for each of the ten stimuli and expressed as a percentage of that time. As well as examining behaviour within the 5 seconds while the stimulus was on, the 5 second pre- and post-stimulus periods were also examined to assess whether the animals were affected by the stimulus when it was not actually on. That is, if their behaviour was a general behaviour directed towards the stimulus object rather than the actual sensory stimulus (i.e. the light), that is a result of arousal rather than attention. The duration data was checked for normality using the Kolmogorov–Smirnov test and then repeated measures ANOVA with STIMULUS PRESENTATION as the within-subjects factor and STRAIN as the between-subjects factor was conducted using the percentage of overall time distracted by the stimulus as the dependent variable. Where Mauchly's test of sphericity was significant in the ANOVAs, the

degrees of freedom were adjusted using Greenhouse–Geisser correction (Greenhouse and Geisser, 1959).

In order to ensure that the measure of orienting was not confounded by locomotor activity differences between the three strains, locomotor activity was measured using automated Activity Monitoring Chambers (Med-Associates, Middlesex, UK). As with the visual response task, testing was conducted over three consecutive days. On the first two days, animals were habituated to the locomotor chambers for 15 minutes each day before an assessment of locomotor activity during a 30 minute (with 5 minute bins) period on the third day. The following measurements were used for analysis (i) "distance travelled" - the total horizontal distance moved in cm; (ii) "average velocity" - average horizontal velocity in cm/min; (iii) "vertical activity"- the number of continuous vertical beam breaks indicating rearing and (iv) "stereotypic activity"- the number of partial-body movements that occurred within a defined space, such as grooming, head-weaving or scratching movements. This locomotor testing took place within 7 days of the orienting task. All variables were checked for normality using the Kolmogorov-Smirnov test and repeated measures ANOVA with TIME as the within-subjects factor and STRAIN as the between-subjects factor was used to analyse locomotor activity for the four different measures. As with the main behavioural data, where Mauchly's test of sphericity was significant in the ANOVAs, the degrees of freedom were adjusted using Greenhouse–Geisser correction (Greenhouse and Geisser, 1959).

2.3 Electrophysiological Recordings

Animals were anaesthetised by an intraperitoneal injection of 30% urethane solution (1.5 g/Kg given in a volume of 5 ml/kg, Sigma Aldrich, Gillingham, UK.). Anaesthetic depth for surgery was assessed by loss of the pedal reflex and eye blink reflex before the animal was placed in a stereotaxic frame (Kopf Instruments, Tujunga, USA) in the skull flat position. Body temperature was measured throughout the experiment using a rectal thermometer

connected to a thermostatically-controlled heating blanket (Harvard Apparatus Ltd, Edenbridge, UK) to maintain temperature at 36-38 ˚C. Both eyes were sutured open and liquid tear gel (Viscotears ®, Novartis Pharmaceuticals Ltd., Surry, UK) applied to prevent desiccation. Following application of local anaesthetic (Ethyl Chloride BP, Cryogesic ®, Acorus Therapeutics Ltd., Chester, UK), scalp retraction, bilaterial craniotomy and durotomy were performed, creating two 3 mm Ø burr holes exposing the cortex above the superior colliculus (right:-6.3 mm AP to Bregma, and +2 mm ML to the midline; left: -6.3 mm AP to Bregma; and +3.5 mm ML to the midline) to allow for simultaneous recordings from both SCs. In addition, two trepanned holes (1 mm Ø) were created anterior to the SC burr holes at specific stereotaxic co-ordinates for electroencephalographic (EEG) recordings (+1 mm anterior, +2 mm lateral; and -4mm posterior, +3mm lateral, relative to Bregma, Devonshire et al., 2009). Differential and active EEG electrodes (loop-tipped silver wire, 0.2 mm Ø; Intracel) were placed ~1 mm subcranially into the rostral and caudal trepanned holes, respectively to obtain continuous EEG information. Finally, respiration rate was recorded using a three-axis accelerometer IC (ADXL330KCPZ, Analog Devices, Norwood, MA, USA), device attached to the animal's lateral abdomen (Devonshire et al., 2009). Both EEG and respiration rate were used to monitor the animal during the recordings and used offline to confirm there were no differences in anaesthetic depth between the three strains.

Tungsten electrodes (Parylene-C-insulated; 2 MΩ, A-M Systems Inc., Carlsborg, WA, USA) were positioned directly above the superficial layers of the SC at the coordinates stated above at a depth of – 2.0 mm from the brain surface. The electrodes were then gradually lowered during presentation of a light stimulus (green LED flashing at 0.5 Hz, 10 ms duration, 20 mcd positioned 5 mm anterior to the contralateral eye) until a strong light response was detected in both the audio feed from the recording (NL120, The Neurolog System, Digitimer, Hertfordshire, UK) and visual feed via Spike2 (CED, Cambridge, UK.). Once the electrodes

were positioned in the superficial layers, the animal was left in the dark for a further 25 minutes to adapt to the darkness before actual recordings began. Visual responses from 150 stimulations were then recorded at 5 different stimulus intensities (from minimum to maximum light: 4, 8, 12, 16 and 20 mcd) for offline analysis. Extracellular low frequency (local field potential; LFP) and high-frequency (multi-unit activity) was amplified (gain 10,000 and 1000, respectively), band-pass filtered (LFP: 0.1–500 Hz, multi-unit activity: 500–10 kHz) (Logothetis, 2008), digitized at 11 kHz and recorded to PC using a 1401+ data acquisition system (Cambridge Electronic Design Systems, Cambridge, UK), running Spike2 software (Cambridge Electronic Design, Cambridge, UK) and saved for offline analysis.

To check that the depth of anaesthesia was comparable in the three strains during testing, the dominant EEG frequency was obtained using a power spectrum analysis (Spike2) for the period within which the 150 stimulations where presented. The respiration rate per minute was calculated during the first and last 30 seconds of this period and then used to calculate an average rate per minute over the whole recording period. Based on the EEG frequency bands, all animals were found to be in stage III-4 (Guedel, 1920), with a dominant EEG frequency of 1-2 Hz during recordings, and were found to have comparable respiration rates using a One-Way ANOVA ($F(2) = 3.52$; $p = 0.098$) following confirmation of normality of data with a Kolmogorov–Smirnov test.

Collicular recordings were analyzed offline using Spike2, custom-made Excel macros (Peter Furness, Sheffield University) and SPSS. All analyses were performed on averaged data where averages were constructed from the full 5 minute period (150 stimulations) for each of the five stimulus intensities. The main comparison of interest was between responses in the three strains across the range of flash intensities. For LFP data a waveform average was created in Spike2 (1-ms bins, 1 s duration, 0.1 s offset) for each intensity. The waveform average was exported into the custom-made macro and a response was deemed to have

occurred if the voltage trace exceeded a pre-determined threshold after stimulus onset, but not before 20 ms post stimulus. The latter requirement was used to avoid any stimulus-related artifacts; collicular LFP responses to light flash stimuli in dark-adapted rats have been reported to have an average onset latency in excess of 27 ms (Dyer and Annau, 1977, Gowan et al., 2008). The threshold for change was set at ±1.96 standard deviations from the mean baseline (i.e. within 95% confidence levels). This threshold was used to assess three parameters: onset latency, peak-to-peak amplitude and duration. Onset latency was obtained by recording the time after stimulus presentation (and at least 20 ms) at which the voltage trace exceeded the threshold. Response duration was determined by obtaining the time, post-stimulus, when the voltage trace returned to within baseline levels (i.e. ±1.96 standard deviations of the pre-stimulation mean) and consistently stayed below this value for 10 ms or 10 bins. The time between onset latency and the response finishing was then used to calculate duration. Finally, peak-to-peak amplitude was defined as the voltage difference between the maximum positive peak and the maximum negative peak in the response period defined by the significant deviation from baseline. For the multiunit activity, similar measures were utilised following initial extraction of 'spikes' from the high-frequency data by thresholding. Peri-stimulus time histograms (PSTHs; 1-ms bins, 1 s duration, 0.1 s offset) were constructed from the trial-by-trial spike counts within Spike2 and the 100 ms pre-stimulus period was defined as baseline activity. A light response was deemed to have occurred if, post stimulus, the activity rose above 1.96 standard deviations of the mean for at least 5 ms (5 consecutive bins), the first of which was labelled as the onset of a response. The duration was calculated by measuring when the response fell back to within the baseline levels for at least 10 ms (10 consecutive bins), the first of which was labelled as the end of the response. Duration was then given as the difference between onset latency and the response ending. The amplitude was recorded as the peak value of the response minus the mean baseline value. Prior to

statistical analysis all data were deemed normally distributed using the Kolmogorov–Smirnov test. Repeated measures ANOVAs with STRAIN as the between-subjects factor and STIMULUS INTENSITY as the within-subjects factor were used. As with the behavioural data, where Mauchly's test of sphericity was significant in the ANOVAs, the degrees of freedom were adjusted using Greenhouse–Geisser correction (Greenhouse and Geisser, 1959).

2.4 Histology

Site reconstruction: Following electrophysiological recordings, animals were transcardially perfused with physiological saline followed by 4% paraformaldehyde in phosphate buffered fixative. The brains were the placed in fixative for 24 hours before being transferred to 20% sucrose for a further 36 hours. They were then frozen to -18℃ in isopentane (WWR International, Lutterworth, UK) and cut into 50 µm coronal sections using a cryostat (CM1900, Leica, Milton Keynes, UK) with the cutting chamber held at -20℃. The slices were dehydrated with alcohol and Nissl stained with cresyl violet (0.5%) (Sigma Aldrich, Gillingham, UK), before cover-slipping for histological verification of recording sites, which were subsequently plotted onto reconstructed sections from Paxinos and Watson (Paxinos and Watson, 1998) to confirm location of recording in the superficial layers of the colliculus.

Collicular volume and cell counts: Animals were given a terminal i.p. dose of sodium pentobarbitone (Animalcare, York, UK) before being trancardially perfused and the brain sectioned as described above. For volume analysis of the whole brain and the SC the Cavalieri principle was used; the first 50 µm section was taken from every 1-in-5 series of sections throughout the brain were used. For the cell counts, beginning at a random starting point (between slices 1-5), every 5^{th} section was collected for cell count analysis. For both measures the slices were dehydrated with alcohol and Nissl stained with cresyl violet (0.5%, Sigma Aldrich, Gillingham, UK) before cover-slipping for analysis. Images were captured

using a Microfibre digital camera attached to a Nikon Eclipse 80i microscope (Nikon UK LTD, Kingston-upon-Thames, UK). For volume analysis, images of the section and an appropriate scale bar were taken at x1 magnification (Nikon Plan UW, 1x/0.04, WD 3.2) and exported to a freely available reconstruction programme (Reconstruct version 1.1.0.1 http://synapses.clm.utexas.edu/). The whole brain as well as the complete superficial layers of the SC, as defined by Paxinos and Watson (Paxinos and Watson, 1998), were then outlined throughout the slices using the Reconstruct programme. The multiplication of the cut surface area by the known distance in thickness (250 μm) was calculated to provide the estimated volume of the examined objects i.e. the whole brain and the superficial layers of the colliculus. Factors such as the physical size of the animal influence the maximum brain size (Raz et al., 1998) and it has therefore been suggested that comparing solely volumes of intracranial structures between groups would not provide reliable data (Knutson et al., 2001). As such, the volume fraction of the SC within the reference volume (the whole brain) was calculated, to give a proportion of the structure (i.e. superficial layers) within the whole brain structure. These data were confirmed as having a normal distribution using the Kolmogorov–Smirnov test before analysis was conducted using a One-Way ANOVA to analyse strain differences. For cell counts, the images were taken at x40 magnification (Nikon Plan Flor, 40x/0.75, DIC M, WD 0.72). Contours were drawn at low magnification (x1; Nikon Plan UW, 1x/0.04, WD 3.2) around the region of interest i.e. the superficial layers of the SC, as defined by Paxinos and Watson (1998). The stereologically unbiased Optical Fractionator method on the Stereo-Investigator software (MBF Biosciences, Magdeburg, Germany) was used to obtain an estimate of the total number of cells in the region of interest, as it is not influenced by the size, shape, spatial orientation, and spatial distribution of the cells studied. Nuclei from different cell types were differentiated based on morphological criteria of shape and relative size (see Figure 1). Neurons were identified by their generally larger shape and

non-spherical outline, as well as a pale and uniformly Nissl-stained cytoplasm with a well-marked nucleolus. Glial nuclei were identified by being generally smaller in size, ovoid shape with the absence of stained cytoplasm, the presence of a thicker nuclear membrane, and more heterogeneous chromatin within the nucleus (Cotter et al., 2002). Although we cannot definitively distinguish between glial types, on the basis of their appearance we believe that the large majority are astrocytes.

Figure 1 Examples of cresyl violet stained neuronal and glial cells in the superior colliculus at x40 magnification, differentiated based on specific morphological criteria. N: neuron; G: glia. Scale bar = 10µm.

3. Results

3.1 The SHR showed less habituation and longer duration responses to visual stimuli.

The vast majority of animals (100% SHR, 100% WKY and 88% WIS) responded to the visual stimulus on the first presentation, as expected for a novel stimulus. However, although the percentage of animals responding for the three strains decreased with repeated stimulus presentation, the rate at which the responding decreased differed between strains (Figure 2A), with 66.67% of SHR still responding to the final stimulus presentation in comparison to just 11.11% of WKY and none of the WIS. The survival analysis showed a significant difference in median survival time between strains ($U(2)=28.96$; $p<0.001$), with post-hoc analysis revealing that the SHR (median survival time = 10.00) was significantly more likely to respond for longer than the WIS (median survival time = 8.31; $U(1)=26.39$; $p<0.001$) and WKY (median survival time = 8.37; $U(1)$ 27.33; $p<0.001$). There was no significant difference between the WIS and WKY ($U(1)=0.044$; $p=0.835$). In the 5 second periods either side of the stimulus light being on, animals were not responsive to the stimulus object and this remained the case for all stimulus presentations.

In terms of response duration, all three strains spent a similar amount of time responding to the stimulus during the first presentation (Figure 2B; SHR 67.56±8.97% of total time or 2.88±0.45 s; WIS 52.50±9.13% or 2.63±0.48 s; WKY 54.89±6.19% or 2.74±0.31 s). Repeated measures ANOVA with STIMULUS PRESENTATION as the within-subjects factor and STRAIN as the between-subjects factor was conducted using the percentage of overall time responding to the stimulus as the dependent variable. There was a significant main effect of STIMULUS PRESENTATION ($F(5.67, 130.55)=13.38$; $p<0.001$), with all animals spending significantly less time responding to the stimulus with repeated stimulus presentation, with significant decreases in response duration compared to the first stimulus

beginning at the second stimulus (F (1, 23) =6.82; p=0.016). By the final stimulus there was a highly significant difference in the duration of their response (F (1, 23) =70.29; p<0.001) relative to the first stimulus presentation. There was also a significant main effect of STRAIN (F (2, 23) =56.05; p<0.001), with post hoc tests showing that the SHR spent significantly more time responding to the stimulus than the WIS (p<0.001) and the WKY (p<0.001). There was no significant STIMULUS PRESENTATION x STRAIN interaction (F (11.35, 130.55) =0.627; p=0.808).

Figure 2 The percentage of animals responding to consecutive light flashes (A) and the duration of responses as a percentage of the five second period in which the light was on (B). The SHR showed greater duration responses and continued to respond for a greater number of stimulus presentations. A representative key is shown in part A.

Repeated measures ANOVA with TIME as the within-subjects factor and STRAIN as the between-subjects factor was used to analyse locomotor activity for the four different measures (distance travelled, vertical activity, average velocity and stereotypic activity; Figure 3) in order to be sure that locomotor activity did not confound measures of distractible behaviour. There was no main effect of STRAIN for average velocity (F(2, 23)=0.66; p=0.528) or stereotypic activity (F(2, 23)=0.44; p=0.650). However, there was a main effect of STRAIN for distance travelled (F(2, 23)=4.10; p=0.030), with post hoc (Tukey HSD) analysis revealing that there was a trend towards the WKY moving significantly less distance than the WIS (p=0.052) and SHR (p=0.056), and no significant difference between the WIS and SHR (p=0.994). There was also a main effect of STRAIN on vertical activity (F(2, 23)=4.12; p=0.029), with post hoc (Tukey HSD) analysis showing that the SHR were significantly more vertically active than WKY (p=0.023) but not the WIS (p=0.480). There were no significant differences between WIS and WKY (p=0.265). As may be expected for locomotor activity in a confined space, there was a main effect of TIME, with parameters decreasing with increasing time within the chamber as the environment became familiar through exploration, for distance travelled (F (5, 115)=67.46; p<0.001), stereotypic activity

(F(5, 115)=31.57; p<0.001) and vertical activity (F(3.07, 70.71)=11.02; p<0.001). There was no main effect of TIME on average velocity (F(3.63, 83.41)=2.38; p=0.064). There were no significant TIME x STRAIN interactions for average velocity (F(7.25, 83.41)=1.02; p=0.423), stereotypic activity (F(5, 115)=1.63; p=0.125) and vertical activity (F(6.15, 70.71)=2.08; p=0.070). There was a TIME x STRAIN interaction for the distance travelled (F(10, 115)=5.15; p<0.001). Restricted ANOVAs revealed this significant interaction to be due to differences between the WIS and the other two strains in the first ten minutes with the WIS showing a greater decrease during this period.

Figure 3 Locomotor activity for all three strains. There was a main effect of time and strain on distance travelled (A) and vertical activity (C) but not on average velocity (B). For stereotypic activity (D) there was a main effect of time but not strain.

3.2 The SHR is more likely to show multiunit visual responses to weaker stimuli and have delayed onset of responses.

Eighty-six visual responses were recorded from the superficial layers of the SC (Figure 4); 32 were recorded in Opticum (Op), (12 SHR; 10 WIS; 10 WKY), 53 were recorded in Superficial Grey (SuG) (15 SHR; 20 WIS; 18 WKY) and 1 was recorded from Zonal Layer (Zo) (WKY). Chi-square analysis showed there was no significant association between strain and the superficial layer from which recordings were made ($\chi^2(4)= 2.81$; p=0.590).

Figure 4 Reconstructed plots of recording sites in the superficial layers of the SC for SHR (black circles), WKY (grey circles) and WIS (grey triangles). Plots are collapsed onto three sections through the colliculus (Paxinos and Watson, 1998) with position relative to Bregma given. There was no significant association between the layer recorded from and strains.

The percentage of animals showing responses at each of the five stimulus intensities is shown by strain in Table 2. Chi-square analysis showed that there was no significant association between strain and the likelihood of a response at any of the stimulus intensities for LFP responses, indicating all three strains were equally responsive in terms of LFP responses. By contrast, the likelihood of showing a multiunit activity response was significantly associated

with strain for 4, 8 and 12 mcd stimuli. Restricted Fisher's Exact tests revealed that the SHR was more likely to respond than the WIS at all three intensities (4 mcd p=0.003; 8 mcd p=0.006; 12 mcd p=0.005) and more likely than the WKY to respond to the lowest two intensities (4 mcd p=0.018; 8 mcd p=0.025; 12 mcd p=0.237). There were no differences between the WIS and the WKY at any intensity (4 mcd p=0.589; 8 mcd p=0.606; 12 mcd p=0.181).

Table 2 Responsiveness to the five different stimulus intensities used for whole field light flashes. There were no differences in responsiveness of strains for local field potential responses but for multiunit activity responses, the SHR were more responsive at lower intensities.

Local field potential responses: In order to analyse the impact of stimulus intensity on onset latency, peak-to-peak amplitude and duration, data from the animals that responded to the highest three stimulus intensities were analysed (SHR n=25; WKY n=23; WIS n=23) using repeated measures ANOVA with STIMULUS INTENSITY as the within-subjects factor and STRAIN as the between-subjects factor. These analyses revealed a significant main effect of STIMULUS INTENSITY ($F(1.73, 117.59)=27.62$; $p<0.001$) for onset latency, with significant decreases between each consecutive stimulus intensity (12-16 mcd: $F(1, 6)=10.09$; $p=0.002$), 16-20 mcd: $F(1)=52.11$; $p<0.001$), but no significant main effect of STRAIN ($F(2, 68)=0.32$; $p=0.730$) or STIMULUS INTENSITY x STRAIN interaction ($F(3.46, 117.59)=2.23$; $p=0.080$) (Figure 5B). Similar patterns were found for peak-to-peak amplitude with a significant main effect of STIMULUS INTENSITY ($F(1.11, 75.71)=33.47$; $p<0.001$), with significant increases between each consecutive stimulus intensity (12-16 mcd: $F(1, 68)=23.16$; $p<0.001$), 16-20 mcd: $F(1)=34.41$; $p<0.001$), but no significant main effect of STRAIN ($F(2, 68)=0.006$; $p=0.994$) or STIMULUS INTENSITY x STRAIN interaction ($F(2.23, 75.71)=1.40$; $p=0.252$) (Figure 5C). The duration of the responses is shown in Figure 5D. Unlike the other parameters, there was no significant main effect of STIMULUS INTENSITY ($F(2, 136)=0.81$; $p=0.447$), but there was a significant main effect of STRAIN

(F(2, 68)=6.07; p=0.004). Post hoc (Tukey HSD) analysis revealed that the WKY had a significantly longer response duration in comparison to the SHR (p=0.013) and WIS (p=0.007). There was no significant STIMULUS INTENSITY x STRAIN interaction (F(4, 136)=0.52; p=0.734).

Figure 5 Example of a local field potential visual response recorded in the superficial layers of the superior colliculus with a 20 mcd stimulus in an SHR, with the stimulus presented at time zero (grey line) (A). The relationship between local field potential response parameters and stimulus intensity is shown for onset latency (B), peak-to-peak amplitude (C) and duration (D). A representative key is shown in B.

Multiunit activity responses: As with the LFP responses, in order to analyse the impact of stimulus intensity on stimulus parameters of onset latency, peak amplitude and duration, data from the animals that responded to the highest three stimulus intensities were analysed (SHR n=27; WIS n=22; WKY n=26) using repeated measures ANOVA with STIMULUS INTENSITY as the within-subjects factor and STRAIN as the between-subjects factor. For onset latency, there was a significant main effect of STIMULUS INTENSITY (F(1.43, 109.59)=59.79; p<0.001), with significant decreases in latency with each consecutive increase in stimulus intensity (12-16 mcd: F(1, 72)=20.90; p<0.001, 16-20 mcd: F(1, 72)=133.31; p<0.001). However, there was also a significant main effect of STRAIN (F(2, 72)=4.06; p=0.021). Post hoc (Tukey HSD) analysis showed that the SHR had a significantly greater onset latency in comparison to the WKY (p=0.022). There was no significant STIMULUS INTENSITY x STRAIN interaction (F(2.85, 102.59)=0.56; p=0.637) (Figure 6B). For peak amplitude there was a significant main effect of STIMULUS INTENSITY (F(1.68, 120.68)=130.15; p<0.001), with significant increases in amplitude with each consecutive stimulus intensity (12-16 mcd: F(1, 72)=59.00; p<0.001, 16-20 mcd: F(1, 72)=167.75; p<0.001), but no significant main effect of STRAIN (F(2, 72)=1.26; p=0.298). There was however a significant STIMULUS INTENSITY x STRAIN interaction (F(3.35, 120.68)=3.79; p=0.010) for this parameter. Restricted ANOVAs indicated that this

interaction was due to the SHR having a greater amplitude responses at the 12 and 16 mcd intensity but not at the 20 mcd intensity (Figure 6C). The duration of the responses is shown in Figure 6D. Unlike the duration of the LFP response, there was a significant effect of stimulus intensity ($F(1.59, 114.57)=38.01$; $p<0.001$), with consecutive increases in stimulus intensity associated with significant increases in response duration (12-16 mcd: $F(1, 72)= 40.43$; $p<0.001$, 16-20 mcd: $F(1, 72)=37.06$; $p<0.001$). There was also a significant main effect of STRAIN $F(2, 72)=5.27$; $p=0.007$), with post hoc (Tukey HSD) analyses revealing only one significant difference - the WKY had a longer response duration than the WIS ($p=0.005$). There was no significant STIMULUS INTENSITY x STRAIN interaction ($F(3.18, 114.57)=0.44$; $p=0.740$).

Figure 6 Example multiunit visual responses recorded in the superficial layers of the superior colliculus of an SHR at the 12 mcd intensity The top trace shows a raster plot with a line for each trial whilst the lower trace is a histogram (1 ms bins) of spike activity with the stimulus presented at time zero (grey line) (A). The relationship between multiunit response parameters and stimulus intensity is shown for onset latency (B), peak-to-peak amplitude (C) and duration (D). A representative key is shown in B.

3.3 There were no significant differences in collicular volume fraction or cell densities but glia:neuron ratio varied with strain.

Despite there being no significant differences in body weight between the three strains for these experiments, analysis of whole brain volume using a One-Way ANOVA revealed a significant difference between the three strains ($F(2)=6.77$; $p=0.016$), making it necessary to normalise collicular measures to whole brain volume. A One-Way ANOVA using volume fraction to assess the volume of the superficial layers of the SC revealed that there was no significant difference ($F(2)=1.04$ $p=0.392$) between the strains (Figure 7A). There were also no significant differences in number (Figure 7B $F(2)=0.58$; $p=0.57$) or the density of neurons (Figure 7C $F(2)=1.40$; $p=0.279$) between the strains. There was a significant difference in the number of glia (Figure 7B $F(2)=4.23$; $p=0.037$) with post hoc (Tukey HSD) tests revealing

that the SHR had significantly fewer glia than the WKY (p=0.038) but not the WIS (p=0.126). There was no significant difference between the WIS and WKY (p=0.853). When density of glia was considered, therefore taking into account the differing brain absolute volumes, there was no significant difference between strains (Figure 7C F(2)=0.56; p=0.582). There was, however, a significant difference between strains in terms of the glia:neuron ratio (Figure 7D; F(2)=3.80; p=0.048). Post hoc (Tukey HSD) tests revealed that the SHR had a significantly lower ratio than the WKY (p=0.047) but not the WIS (p=0.167). There was no significant difference between the WIS and WKY (p=0.825).

Figure 7 There was no significant difference between the three strains in terms of superficial collicular volume fraction (A). Whilst there was a significant strain difference for number of glia, but not neurons (B), this was not present when taking into account absolute volume differences by using cell densities (C). However, the SHR had a significantly lower glia:neuron ration than the WKY (D) * p<0.05. A representative key for C and D is shown in C.

4. Discussion

The finding that the male SHR used in the current studies were more responsive than both WKY and WIS rats to a visual stimulus, and that they fail to habituate to repeated presentation, is in line with a previous study using a similar orienting task in female SHR (Robinson and Bucci, 2014). Therefore, despite other recorded sex differences in SHR behaviour (Berger and Sagvolden, 1998, Ferguson et al., 2003, Dervola et al., 2012, Johansen et al., 2014), increased responsiveness on an orienting task appears common to both sexes. It noteworthy that the SHR have been found to show reduced habituation (Hendley et al., 1985) and increased locomotor activity at some ages (Bayless et al., 2015) when compared to WKY and alterations in either of these could have confounded our results. However, activity during the periods immediately before and after the visual stimulus and patterns of activity in the

locomotor activity monitoring chambers does not support this and, therefore, we suggest that neither of these factors confounded our results.

Also in line with previous studies (Gowan et al., 2008, Clements et al., 2014) we recorded complex LFP responses in the superficial layers of the SC in response to whole field light flashes. These LFP responses had an onset latency that varied from 58-80 ms in the SHR, 55-80 ms in the WKY and 53-77 ms in the WIS, with the fastest latencies for all strains at the higher intensity stimulus, consistent with other studies (Dyer and Annau, 1977, Clements et al., 2014). Similarly, the peak-to-peak amplitude at the highest stimulus intensity (105-135 µV; see Figure 3) was in line with previous work (Clements et al., 2014). The duration of the LFP response at the highest intensity for the SHR (mean = 223 ms) and WIS (mean =229 ms) rats was also consistent with previous studies (Gowan et al., 2008, Clements et al., 2014), although the duration of response in WKY (mean = 372 ms) was significantly longer than the other two strains. Interestingly, there were no significant differences between the SHR and both the WIS and WKY for any parameters for the LFP responses, which suggests that the SHR did not differ from control strains in terms of the visual information entering the colliculus, which would likely be seen in a change to the LFP response, as the LFP response is best described as representing 'peri-synaptic activity', which includes post-synaptic potentials (Logothetis, 2008, Ekstrom, 2010). This finding is in direct contrast to the results of a similar study on the New Zealand GH rat, which is also a proposed, but not yet widely-validated, model of ADHD. Clements et al. (Clements et al., 2014) found increased visual LFPs in the GH rat at a range of stimulus intensities. Although we did not find increased LFP responses in the SHR, we did find that the SHR were more likely to produce a multiunit response than both control strains for lower stimulus intensities. Furthermore, the amplitude of the response tended to be greater in the SHR at moderate intensities. It is likely that a ceiling effect prevented this remaining at the higher intensities. SHR responses also showed

delayed onset latency, relative to both other strains. These multiunit responses are partially consistent with Clements et al. (Clements et al., 2014) because they also reported an increase in multiunit responses in the GH rat, however, they found reduced latency (albeit peak rather than onset). The selective increase in spiking (mulitiunit) activity rather than LFP responses in the SHR suggests that any alteration to function is confined to the SC itself rather than being the result of these local changes combined with an alteration in afferent driving as may be the case in the GH rat. Furthermore, a delayed response onset is consistent with ADHD being a developmental disorder because it is known that during development transmission within the retinocollicluar pathway increases in speed, giving quicker onset latencies within the SC as this system develops (Reece and Lim, 1998, Crognale et al., 2001). It is also in line with the observation of significantly longer saccade latencies in visually guided saccades (Mahone et al., 2009, Goto et al., 2010), memory guided saccades (Goto et al., 2010), prosaccades (Klein et al., 2003, Munoz et al., 2003), and antisaccades (Munoz et al., 2003, Feifel et al., 2004, Karatekin, 2006) that has been reported in children with ADHD.

Critically, using recent theories about how specific motor activities are selected, it is possible speculate that in the present study the observed behavioural differences between SHR and the control strains could be directly underpinned by the increased multiunit activity found in the SC. It has been suggested that the SC, amongst other structures, is capable of specifying actions by putting 'bids' into the central selection device, thought to be the basal ganglia (Redgrave et al., 1999). There are a number of routes in which information can travel from the superficial layers of the SC to basal ganglia nuclei (Redgrave et al., 2010). For example, the superficial SC innervates areas of the thalamus that provide the major afferents to the striatum and subthalamic nucleus (STN). These same basal ganglia nuclei can also receive information from the superficial layers via the deep layers of the SC (Takada et al., 1985, Feger et al., 1994, Van der Werf et al., 2002). In addition, the superficial layers also have a

direct tecto-nigral pathway to midbrain dopaminergic cells which provide an important input to the basal ganglia (Comoli et al., 2003). The deeper layers of the SC also have a direct connection to the STN (Tokuno et al., 1994, Coizet et al., 2009) which can convey information from the superficial SC to the STN. Therefore, heightened activity within the SC may have the effect of strengthening the bid to the basal ganglia and increasing the likelihood of the bid 'winning' over competing action choices and generating an output, such as orienting towards a stimulus (Grantyn et al., 2004). Such an increased tendency to respond would result in an increase in distractibility.

We found no difference in the volume fraction of the superficial layers of the SC or in the cell density of neurons and glia within these layers. However, we did find a significant difference in the glia:neuron ratio, with the SHR having a lower ratio, meaning that there were fewer glia for the number of neurons present than in the WKY. This is supported by the cell count data, although it must be recognised that cell count data alone is limited when there are differences in absolute brain volume. Although these findings are preliminary and warrant further investigation, it is possible to speculate about why they may occur in the SHR and how they could contribute to the increased sensory responsiveness found in the SHR.

Glial cells fulfil a broad range of functions and astrocytes in particular participate in a number of interactions that are central to the development, function, and repair of the CNS (Theodosis et al., 2008). They control synapse formation and modulating synaptic activity, responding to and modulating neural activity and responding to sensory stimuli (Herculano-Houzel, 2014). Glia:neuron ratios have received significant attention in recent years and there is now evidence that the ratio increases with the size of neurons rather than the size of the brain as originally believed (Herculano-Houzel, 2014). Two main theories have been suggested as to why the ratio would increase with neuron size. Firstly, it has suggested that increased neuronal size means increased metabolic demands (Hawkins and Olszewski, 1957),

however, data do not support an energy demand model (Herculano-Houzel, 2011). Secondly, it is suggested that the proliferation of glial cells which takes place during postnatal development (Brizzee, 1964), gives rise to a relatively uniform density of glial cells across the brain, the glia:neuron density being largely determined by size (and hence density) of neurons in each structure. A lower ratio in the SHR could therefore suggest smaller neuronal cells in the superficial layers of the colliculus in this strain, which should be investigated in future, along with determining ratio by volume, which is often deemed more useful than a count ratio (Herculano-Houzel, 2014). Another possibility is that normal development, featuring postnatal proliferation of glial cells in the second and third postnatal weeks in rat (Bandeira et al., 2009), is delayed or deficient in the SHR, which is in line with the modelled condition being a developmental disorder. Although this is the first study to report differences in glia:neuron ratio in the SHR and the technique used in the present study does not allow us to discriminate between types of glia, alterations in astrocytic function has been purported to be critical in ADHD (Todd and Botteron, 2001, Killeen et al., 2013), with reduced neural energy available, which would be in line with a decrease in glia:neuron ratio. In addition, the psychostimulants used in the treatments of the disorder have been found to increase astrocyte activation (Bahcelioglu et al., 2009, Narita et al., 2009). A reduction in glia:neuron ratio may reduce energy available for neuronal responses, but it is also likely to impact directly on glutamatergic neurotransmission within the colliculus. One possible way in which it could do this is via Group II mGluR2/3 which have been found to be present on astrocytes within the superficial SC (Cirone et al., 2002a, Cirone et al., 2002b). Activation of these receptors is known to modulate visual responsiveness. Therefore, if fewer receptors are present due to reduced glial cells then, visual responsiveness may be altered. Petralia et al. (Petralia et al., 1996) found that mGluR3 are located at synapse wrappings on glial cells and function to enhance the efficiency of glutamate conversion to glutamine and therefore to terminate

glutamate transmission. If there are few glial cells and therefore mGluR3 receptors fulfilling this function then it is plausible that enhanced visual responses would be found. Certainly expression of these receptors in the colliculus of the SHR should be further investigated.

5. Conclusion

In summary, the present study has demonstrated increased distractible behaviour in the SHR model of ADHD, with reference to two control strains. In addition, we have proposed a plausible physiological basis for this behaviour in the form of increased collicular visual responsiveness. The exact cause of this increased responsiveness has yet to be determined, however, the current study points towards a role for glial-mediated effects.

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Measure	Strain		
	SHR	WIS	WKY
Orienting task and general activity	400±11 (N=9)	448±10 (N=8)	466±14 (N=9)
Recordings of visual responses from superficial colliculus	396±6 (N=27)	490±13 (N=30)	400±9 (N=29)
Morphology: Collicular volume	397±16 (N=4)	485±29 (N=4)	431±28 (N=4)
Morphology: Collicular cell densities and ratios	414±16 (N=6)	456±19 (N=5)	410±28 (N=6)

Table 1

Local Field Potential Responses					
Stimulus intensity (mcd)	Percentage responding			Strain – responsiveness association	
	SHR (n=27)	WIS (n=30)	WKY (n=29)	χ^2 (df=2)	Significance
4	70.4	50.0	48.4	5.05	0.282
8	75.9	73.3	61.3	1.75	0.416
12	92.6	76.7	79.3	2.82	0.244
16	100	96.7	96.6	0.99	0.626
20	100	100	100	N/A	
Multiunit Activity Responses					
Stimulus intensity (mcd)	Percentage of animals responding			Strain – responsiveness association	
	SHR (n=27)	WIS (n=30)	WKY (n=29)	χ^2 (df=2)	Significance
4	70.4	30.0	37.9	10.32	0.006
8	75.9	43.3	51.7	9.24	0.010
12	100	73.3	89.7	9.29	0.010
16	100	93.3	96.6	1.88	0.391
20	100	100	100	N/A	

Table 2

Figure 1

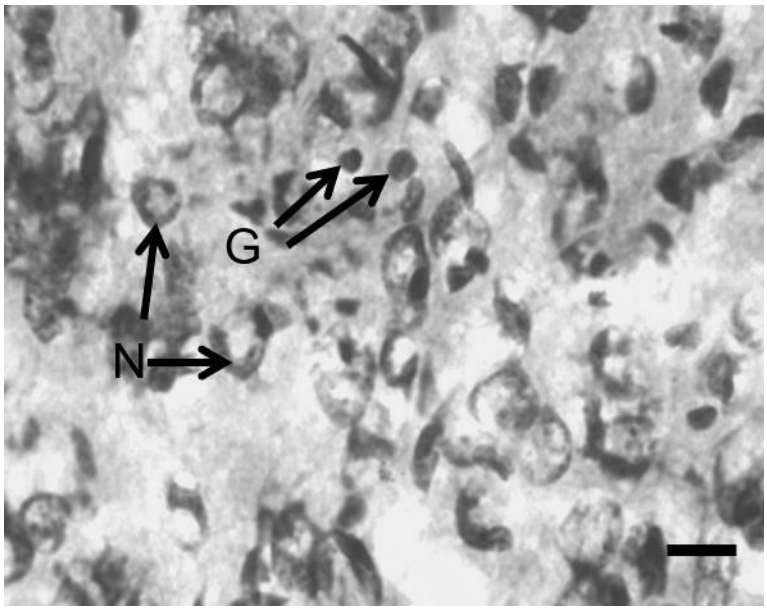


Figure 2

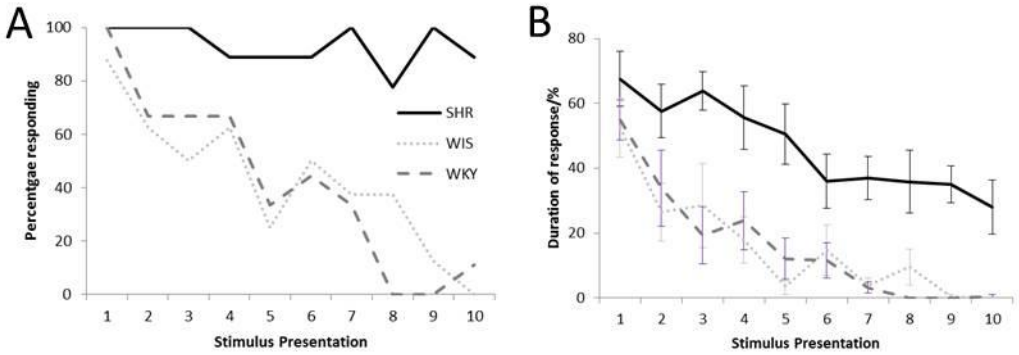


Figure 3

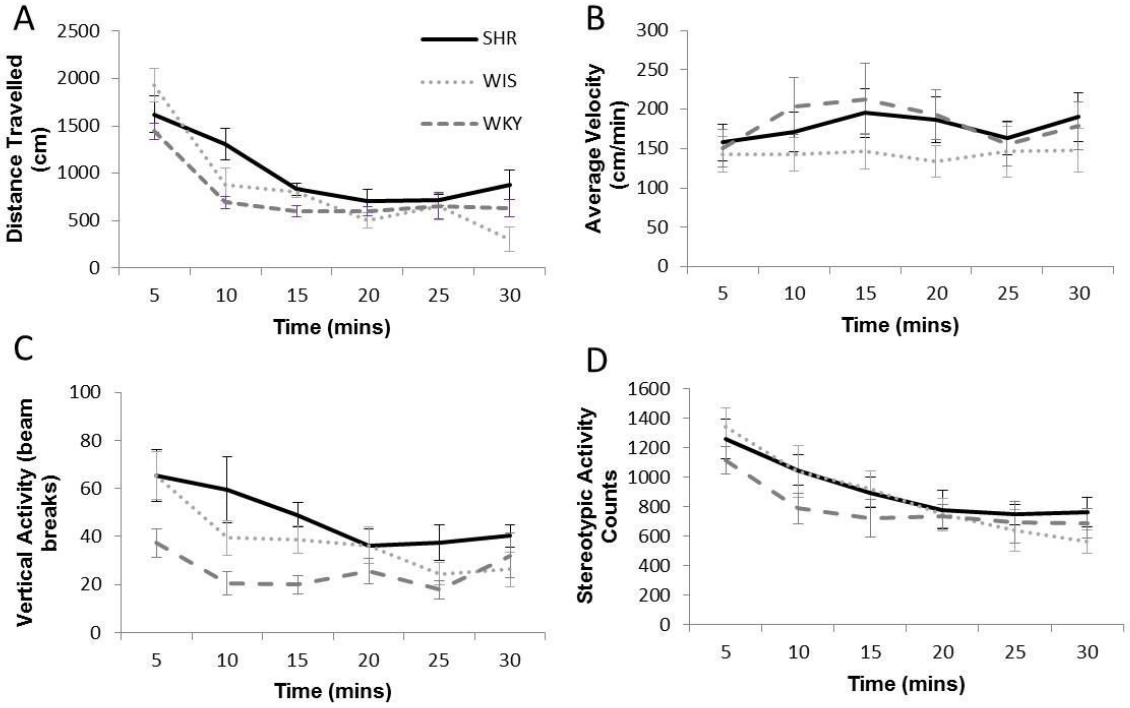

A
Distance Travelled (cm)
2500
2000
1500
1000
500
0
5
10
15
20
25
30
Time (mins)
SHR
WIS
WKY
B
Average Velocity (cm/min)
300
250
200
150
100
50
0
5
10
15
20
25
30
Time (mins)
C
Vertical Activity (beam breaks)
100
80
60
40
20
0
5
10
15
20
25
30
Time (mins)
D
Stereotypic Activity Counts
1600
1400
1200
1000
800
600
400
200
0
5
10
15
20
25
30
Time (mins)

Figure 4

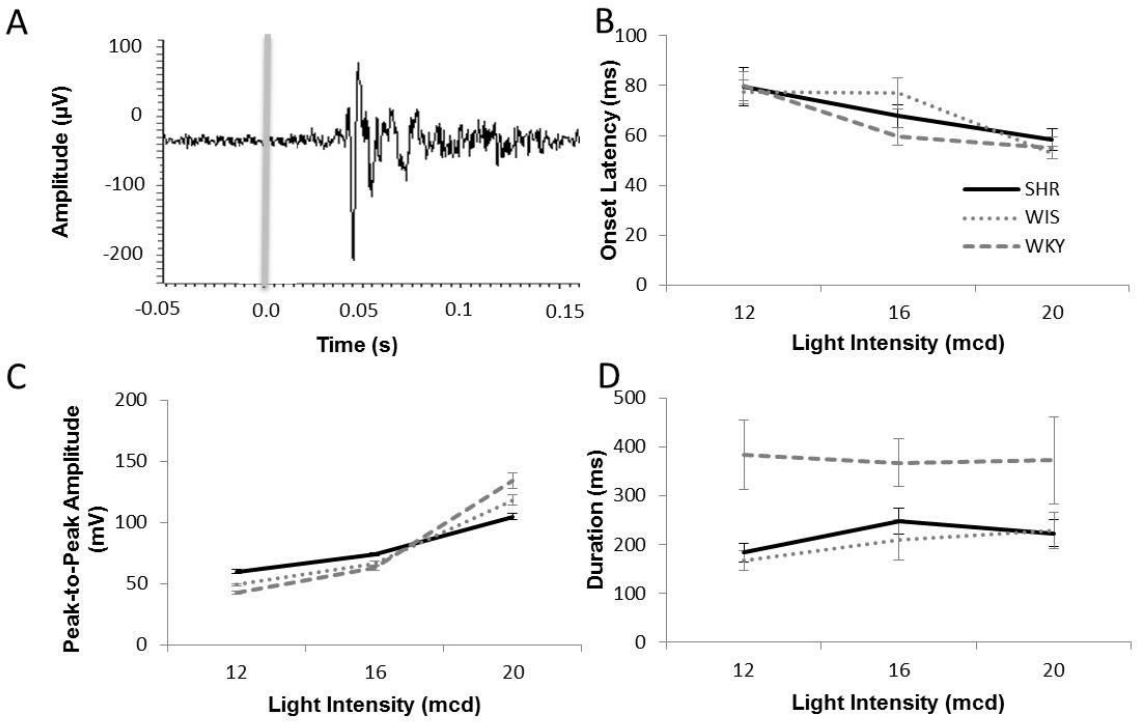

A
Amplitude (µV)
100
0
-100
-200
-0.05
0.0
0.05
0.1
0.15
Time (s)
B
Onset Latency (ms)
100
80
60
40
20
0
12
16
20
Light Intensity (mcd)
SHR
WIS
WKY
C
Peak-to-Peak Amplitude (mV)
200
150
100
50
0
12
16
20
Light Intensity (mcd)
D
Duration (ms)
500
400
300
200
100
0
12
16
20
Light Intensity (mcd)

Figure 5

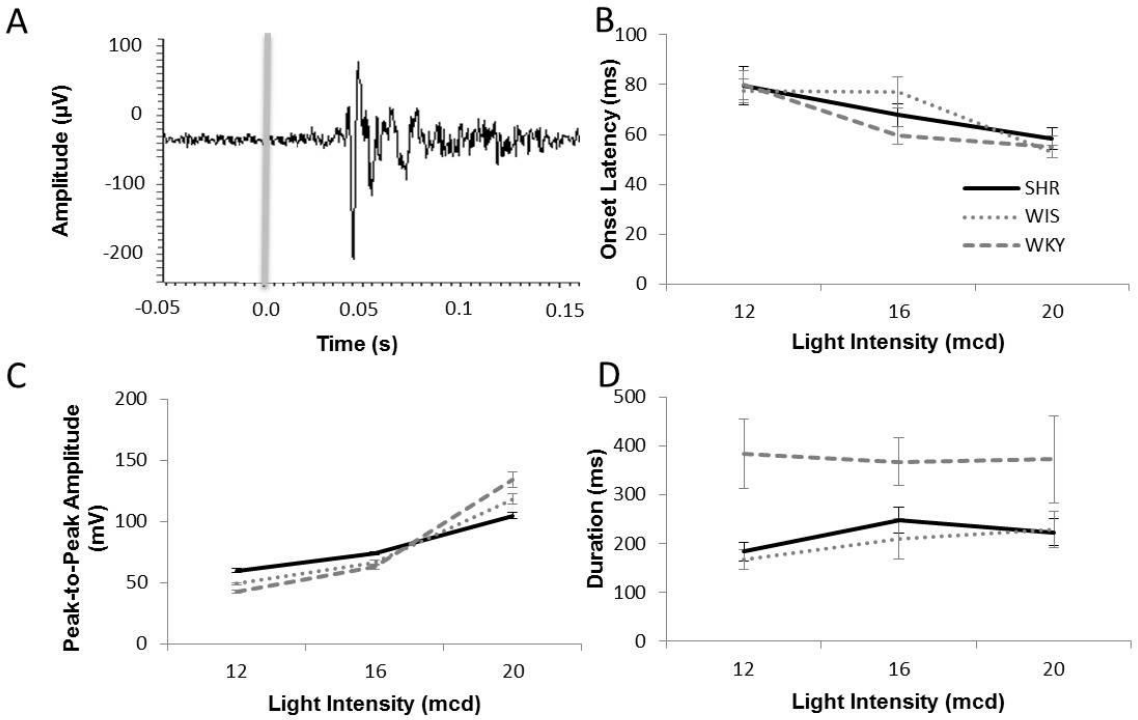


Figure 6

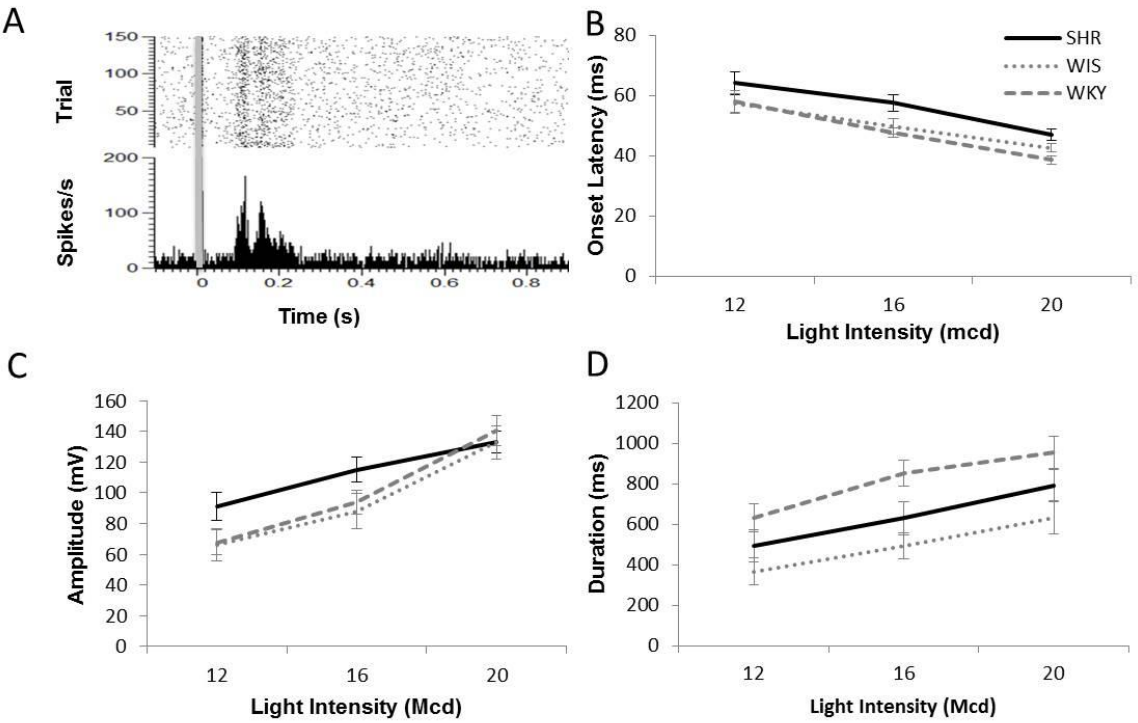
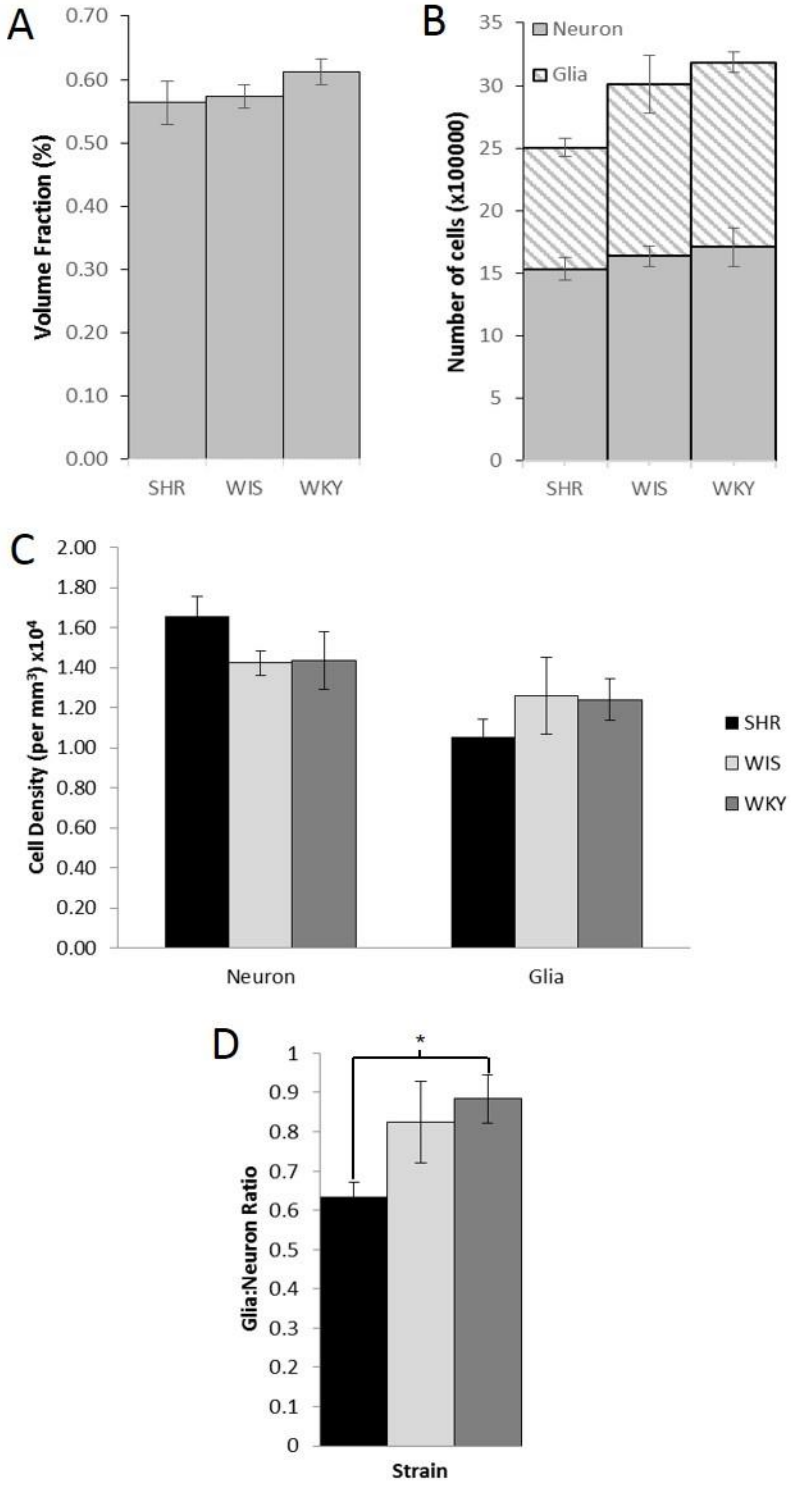

A
Trial
Spikes/s
Time (s)
B
Onset Latency (ms)
Light Intensity (mcd)
SHR
WIS
WKY
C
Amplitude (mV)
Light Intensity (Mcd)
D
Duration (ms)
Light Intensity (Mcd)

Figure 7


A
Volume Fraction (%)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
SHR
WIS
WKY
B
Neuron
Glia
Number of cells (x100000)
35
30
25
20
15
10
5
0
SHR
WIS
WKY
C
Cell Density (per mm3) x10^4
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Neuron
Glia
SHR
WIS
WKY
D
*
Glia:Neuron Ratio
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Strain